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Binding molecules 2

Summary

The present invention relates to a process for making antibodies that bind to a cell receptor and trigger a biological response in the cell, and to antibodies made by this process The binding specificity of the antibody is derived from antibody V-gene repertoires displayed on the surface of a replicable genetic display package (rgdp), in which the rgdp has been selected by binding to a cell receptor.

In this process, the V-gene repertoires are derived from V-genes rearranged in vivo or vitro, and/or by mutation of (a) rearranged V-gene(s). A key feature of the V-gene repertoires is that they must be extremely diverse in sequence, preferably in excess of 10^6 different members. The V-gene repertoires are cloned into the rgdp (for example a filamentous phage vector) such that the antibody repertoires are displayed on the surface of the rgdp. The rgdps encoding rare antibody specificities, such as those binding to cell receptors are selected by virtue of binding to the cell receptors. By cycles of growth and selection, those rgdps binding to the cell receptors are isolated. Some of these rgdps encode binding specificities with the potential (alone or in combination with other binding specificities) to trigger the receptors. These binding specificities are tested alone, or in combination, for triggering the cell receptors.

Source of V-gene repertoires. The rearranged V-genes could be derived from the peripheral blood lymphocytes of a donor, using the polymerase chain reaction and "universal" primers. The DNA or mRNA

could be used as the primary source of rearranged V-genes. and would preferably be derived from the resting B-cell population, for example by selecting populations of surface IgM or IgD B-cells using fluorescent activated cell sorting, or using primers specific for the IgM or IgD cDNA. The rearranged V-genes could alternatively be derived from the in vitro assembly of unrearranged V-genes with artificial D and J segments. Finally a single V-gene could be extensively mutated, for example using error prone polymerases, or synthetic "spiked" oligonucleotides to create extensive sequence diversity.

Replicable Genetic Display Package. The diverse V-gene repertoires are displayed on the surface of an rgdp. An rgdp is a biological particle which has genetic information providing the particle with the ability to replicate. The particle can display on its surface at least part of a polypeptide. The polypeptide can be encoded by genetic information native to the particle and/or artificially placed into the particle or an ancestor of it. The particle may be a virus, for example a bacteriophage.

Displayed antibody repertoires. The V-genes are cloned into the genetic material of the rgdp, and expressed as single domains, for example single heavy chain variable domains, or most preferably as associated antibody heavy and light chain variable domains.

The two domains could be displayed as separate polypeptide chains (linked as in Fab fragments through non-covalent association of domains and/or disulphide bonds), or as part of the same chain (single chain Fv fragments where the two domains are contained within the same polypeptide chain.)

The V-domains could be displayed by fusion of at least one of the V-genes to the gene encoding a surface protein of the rgdp, for example a virion outer coat protein such as the filamentous bacteriophage gene 3 or gene 8 protein.

Alternatively the V-domains could be displayed by binding to the rgdp surface protein. For example disulphide bonds might be forced between the surface protein and the V-domain protein, by introducing a cysteine residue in the surface protein and introducing or using a cysteine residue in the V-domain protein. Thus antibody light chains (encoded by the fd bacteriophage for expression into the bacterial periplasm as soluble fragments) might be linked to the bacteriophage gene 8 protein by introducing a cysteine residue at the N-terminus of the gene 8 protein. As the light chains of the antibody are secreted into the bacterial periplasm, the cysteines may pair with the cysteine of the bacteriophage coat. Thus the phage encoding an antibody fragment displays fragments of the same specificity.

Cell receptors. There are a variety of cell receptors in which the binding of a ligand, for example hormone, growth factor, or peptide triggers a biological event, for example the activation of tyrosine kinase activity, or the opening of an ion channel. The rgdps could be selected for binding to cell receptor (or a related receptor with conserved portions of surface such as from another species), for example by using cells displaying the cell receptor, or using soluble receptor immobilised on solid phase, or using domains or peptide epitopes of the receptor. Ideally the receptor would be provided in a crosslinked form (as required for its triggering).

Triggering of cell receptors. Triggering of receptors at the cell surface seems to involve the relative movement of proteins or subunits. For example in the neurotransmitter-gated receptors, the five subunits that are arranged symmetrically in the membrane plane, delineate an ion pathway down the centre. Binding of the neurotransmitter is thought to alter the size of the central ion channel by causing small rearragments between the subunits in an allosteric transition. For tyrosine kinase receptors, the ligand appears to drive receptor oligomerisation. Thus antibodies with binding specificities directed against a receptor may have the potential to promote an allosteric change or to promote oligomerisation. The oligomerisation of the receptors may also be promoted by using bivalent or bispecific antibodies.

The antibody V-genes encoding the binding activities on the surface of the rgdp can be readily expressed as soluble antibodies or antibody fragments. For example for the fd bacteriophage, by interposing a supressible stop codon between the V-genes and the gene 3 capsid protein, the antibody fragments can be displayed on the surface of the bacteriophage in supressor strains of bacteria, or secreted as soluble fragments in non-supressor strains. Alternatively for expression of complete glycosylated antibodies, the V-genes could be recloned into a eukaryotic expression vector for secretion from myeloma or CHO cells.

The soluble antibodies or antibody fragments may be monovalent fragments, for example single chain Fv fragments or Fab fragments, or bivalent fragments, for example Fab2 or complete antibody fragments. The bivalency could also be promoted in other ways, for example (1) by

encoding a tag, such as a peptide or protein (for example, the subunit of a dimeric protein) that self associates, at the N or C-terminus of the monomeric fragment, (2) using a bivalent antibody that binds to the monovalent fragment, for example to a common C-terminal tag, or to an antibody constant domain (3) chemical cross-linking.

Bispecific antibody or bispecific fragments could also be made as for the bivalent fragments. (For expression of the bispecific antibody or fragment in the same cell, the genes encoding both specificities would need to be introduced together). The different antibody "arms" could be directed against the same receptor, for example to different epitopes, or to two different receptors (to trigger hybrid receptors).

Background to the invention

Antibodies directed against self.

(Cambridge Antibody Technology Limited and Medical Research Council)

It was disclosed in patent application PCT/GB91/01134 that antibody fragments can be displayed on the surface of bacteriophage fd and that they will bind antigen. Antibody fragments can be directly selected using this characteristic. This ability to isolate antibody fragments (Fab, Fv, scFv and VH) using their display on the surface of filamentous bacteriophage has opened up the prospect of the isolation of antibody specificities (i.e. antibodies directed against a particular antigen) that were difficult or impossible to isolate previously. In particular, PCT/GB91/01134 demonstrates that antibody specificities can be isolated from a human who has not been specifically immunised ('unimmunised'), even specificities for antigens such as 2-phenyl-5-oxazolone to which humans will not normally be exposed. In contrast,

screening of a bacteriophage lambda library (where no analogous selection procedure is available) failed to isolate a single antibody against tetanus toxoid from an unimmunised human donor (M.A.A. Persson et al Proc.Natl. Acad.Sci.U.S.A 88 2432-2436, 1991). Antibodies of high specificity have been obtained from phage fd libraries of 'natural' antibodies from unimmunised donors, for instance an antibody against turkey egg lysozyme was isolated with a dissociation constant of 87nM for turkey egg lysozyme which did not cross react with hen egg lysozyme. The possibility therefore exists of the isolation of antibodies to any antigen from a phage library which is sufficiently large and diverse.

A preferred source for the generation of diverse libraries is IgM mRNA. It was found in example 43 of PCT/GB91/01134 that antibody fragments directed against turkey egg lysozyme and 2-phenyl-5-oxazolone were much more readily isolated from a phage library derived from the IgM mRNA from an unimmunised human donor, than from one prepared from IgG mRNA. Furthermore, no 2-phenyl-5-oxazolone binding antibody fragments could be isolated from a library of 2000000 phage antibody clones prepared from IgGmRNA of unimmunised mice (T.Clackson et al, Nature 352 624-628.1991).

For making antibodies against cell surface receptors it is necessary to break tolerance. The simplest anti-self antibodies to isolate should be those from patients with significant concentrations of circulating autoantibodies, due to a breakdown in the tolerance mechanism. The antibodies prepared from V gene libraries will then derive from the mRNA of plasma cells secreting IgG (or IgM) antibody. For instance, anti-self antibodies might be isolated from patients with autoimmune

diseases, for example anti-acetylcholine receptor antibodies would be expected to be isolated from antibody repertoires made from the IgG mRNA of myasthenia gravis patients. For example, an antibody fragment specific for human thyroid peroxidase has been isolated from a bacteriophage lambda library from a patient with thyroid autoimmune disease (S. Portolano et al Biochem. Biophys. Res. Commun. 179 372-377, 1991). This however required extensive screening of 200,000 plaques to obtain one clone. In addition, this library was derived from thyroid tissue, a procedure not readily applicable in most instances. In contrast, the power of selection available using the phage system, demonstrated in PCT/GB91/01134 allows the ready isolation of anti-thyroglobulin autoantibodies from the IgM mRNA of peripheral blood lymphocytes of a donor without disease (Example 1).

However, the vast majority of self antigens do not have associated circulating autoantibodies. In this application, we find that even antibodies against human tumour necrosis factor-α can be isolated from the same library as the antibodies directed against thyroglobulin (Example 2). This opens the prospect of the direct isolation of human antibodies binding to human antigens for a number of purposes such as antibodies which bind to circulating hormones to block, modify or potentiate their action or antibodies that bind to cell surface antigen for imaging or killing for example of cancer cells.

The origin of the V genes that contribute to anti-self antibodies isolated from phage display libraries is not clear. Tolerance to self antigens by the immune system (preventing the generation of antibodies directed against them) is mediated by either clonal deletion or functional inactivation (anergy) of self-reactive B lymphocytes (D.A.Nemazee &

K.Burki Nature 337 562-566, 1989; C.C.Goodnow et al Nature 334 676-682, 1988; S.B.Hartley et al Nature 353 765-769, 1991; D.M.Russell et al Nature 354 308-311, 1991). In either case little circulating anti-self antibody is detectable for most antigens. However, in the case of anergy, functionally inactivated self-reactive cells from the B cell lineage persist in peripheral lymphoid organs leading to B cells in circulation. These rare lymphocytes with anti-self specificity may provide heavy or light chain partners (or even both) for phage antibodies with anti-self specificities. Alternatively, such anti-self specificities may arise from the combination in the library of a VH domain with a VL domain to give a specificity that is normally deleted if it occurs in nature. For this reason, combinatorial libraries and 'chain-shuffled' libraries such as those described in patent applications PCT/GB91/01134 and UK 9120252.3 (Cambridge Antibody Technology Limited) may be a particularly rich source of anti-self antibodies. Whichever of these possibilities applies, a selection procedure of great power, such as that provided by phage antibodies, is required to obtain such rare anti-self antibodies.

The application shows that anti-self antibodies can be isolated from libraries prepared using mRNA derived from peripheral blood lymphocytes. Other sources of such anti-self antibodies may be fetal mRNA or cord blood mRNA (P.M.Lydyard et al Scand J Immunol 31 33-43, 1990) or libraries prepared by the synthetic recombination of V, D and J segments.

B lymphocytes express surface IgM and surface IgD before stimulation with antigen but express little soluble IgM or IgD. These unstimulated cells are more likely to contain antibody genes with anti-self specificities. In contrast, terminally differentiated plasma cells which

secrete soluble antibodies express little surface immunoglobulin. The preparation of cDNA for phage library preparation using primers which are specific for surface IgM or surface IgD will produce a repertoire of antibody genes enriched for the naive, unselected genes. In B lymphocytes which have been functionally silenced by exposure to self there are greatly reduced levels of surface IgM but unchanged levels of surface IgD (C.C.Goodnow et al. supra). Hence, a primer specific for surface IgD may be particularly suitable for isolation of anti-self antibodies.

There is some evidence that B lymphocytes directed against soluble self antigens are functionally silenced and those directed against multivalent membrane bound self antigen are eliminated (S.B.Hartley et al supra; D.M.Russell et al, supra). Thus, the use of synthetic libraries made by VH, DH, JH or VK_JK or VL_JL recombination in vitro or its equivalent may be particularly advantageous for isolation of antibodies directed against multivalent membrane bound self antigens.

Antibodies to trigger receptors

When specific antibodies raised in an animal, including man, by vaccinating the said animal with a specific antigen are themselves used to vaccinate another animal, new antibodies termed anti-idiotypic antibodies (Anti-Ids) are produced able to recognise and bind to the first set of antibodies. Some species of these Anti-Ids are able to mimic the specific biological properties of the original antigen. If for example, the antigen were a peptide hormone or a cell receptor, the Anti-Id to the hormone or cell receptor antigen is able to elicit a response of the cell (See Gaulton, G.N. and Greane, M.I., 1986. Idiotypic mimicry of

biological receptors. Ann. Rev. Immunol. 4,253-280; Sege, K. and Peterson, P. A., 1978., Use of anti-idiotypic antibodies as cell surface receptor probes. Proc. Natl. Acad. Sci. Usa., 75, 2443-2447 for examples.

The essence of current teaching of Anti-Ids as mimics of antigens is that they are produced as a result of constructing antibodies to antibodies of the original antigen.

There is therefore a clear distinction between antibodies prepared by an anti-idiotypic route that mimic antigens such as growth factors or hormones, and antibodies that are made directly to the receptors to trigger the receptors. The antibodies derived by an anti-idiotypic route require the antigen (hormone, growth factor) and will bind to the same epitope on the receptor as the hormone. while the antibodies derived by binding to the receptors need not bind to the same epitope to trigger the receptor. Indeed such antibodies need not mimic a known hormone or growth factor, as their specificity, or binding to receptor (characterised as epitope, on-rate or off-rate) or blood clearance is likely to differ. The process for making the antibodies is also quite different. Anti-idiotypic antibodies are made classically by immunisation of animals. Antibodies directed against self receptors are made by selection from V-gene libraries (as described above).

As well as the advantages over the anti-idiotypic route, the antibodies derived directly by receptor binding may even have advantages over the natural hormone or growth factor. Thus receptors that are defective for binding of the natural hormone or growth factor (for example in a genetic disease), may be triggered by an antibody binding at a different epitope.

As therapeutic agents the various isotypes of antibodies or fragments of antibodies carrying the variable regions responsible for the specificity of the molecule have a number of properties having advantages over the bioactive moiety they mimic. For example, unlike the natural hormones their half-life in circulation can be modified readily. Depending on the antibody isotype or fragment chosen, they have half-lives in circulation in a patient ranging from minutes to several weeks. If long term usage or short term clearance is required this can easily be accommodated by choosing the appropriate antibody isotype without need to use slow release devices as implants, or continuous intravenous infusion etc.

Furthermore, many hormones or tissue growth factors or antigens in general are functionally complex with different epitopes of each of the molecules having various specific functions. Clones of antibody mimics are monofunctional in this respect so could be used to produce one specific biological effect of a hormone without a second effect which latter effect may be disadvantageous to the patient. Thus the lymphokine TNF (tumour necrosis factor) binds to two different classes of cell receptors - one common on vascular endothelial cells, the other common on tumour cells. If the TNF is modified so that it cannot bind to the endothelial cell receptors but can still bind to tumour cell receptors, the tumours are attacked without at the same time inducing the very toxic side effects mediated through the vascular receptors. (This is described in Australian Patent Application PCT/AU90/00337). An antibody mimic able to recognise the tumour cell receptor would be expected to be very specific and kill tumour cells without inducing toxic side effects mediated through the vascular endothelium since it would

have no resemblance to the TNF epitope which binds to receptors on the latter.

Example 1 Isolation of antibody fragments specificities directed against human thyroglobulin from a library of scFv fragments using display on bacteriophage fd

Example 44 of PCT/GB91/01134 describes the selection of antibody scFv fragments directed against thyroglobulin from a library of scFv fragments, derived from unimmunised humans, expresssed on the surface of phage fd were isolated by panning against bovine thyroglobulin. Sixteen clones specific for bovine thyroglobulin were analysed for binding to human thyroglobulin in an ELISA assay as described in example 44 of PCT/GB91/01134. Nine of these clones also bound strongly to human thyroglobulin with absorbance signals of between 1.0 and 1.6 12 minutes after addition of substrate. No cross-reactivity (signal less than 0.05 after 90 min) was found with a panel of unrelated antigens- hen egg lysozyme, BSA, ovalbumin, chymotrypsinogen, cytochrome c, keyhole limpet hemocyanin, insulin, cardiolipin and DNA.

Thus antibodies with specificity for the self antigen, thyroglobulin can be isolated from libraries prepared from unimmunised humans. This procedure using an analogous molecule from another animal to select antibodies and then screening for cross reactivity with the human antigen may be a general approach for the isolation of anti-human antibodies.

Example 2.

Isolation of antibody fragments directed against self antigens from a library of scFvs made from unimmunized blood donors.

Introduction

Naturally occuring V-genes isolated from human PBLs can be constructed into a large library of antibody fragments which contain reactivities against antigens to which the donor has not been exposed (PCT/GB91/01134 ex 42). These libraries may also contain reactivities against self antigens, arising either from self-reactive B-cells which have not been deleted or as non-naturally occuring fragments resulting from VH and VL chain recombination. To test this hypothesis, we panned a large human scFv library displayed on the surface of a phagemid against human TNF-α and a human IgG/κ immunoglobulin.

Methods

Rescue of the library: The library of svFvs was constructed from the RNA of human PBLs and has been previously described (PCT/GB91/01134 ex 42). To rescue phage displaying antibody fragments, approximately 10^9 *E. coli* harbouring the phagemid were used to innoculate 50 ml of 2 x TY containing 1% glucose and 100 µg/ml of ampicillin (2 x TY- AMP-GLU) and grown to an O.D. of 0.8 with shaking. Five ml of this culture was used to innoculate 50 ml of 2 x TY- AMP-GLU, 2 x 10^8 TU of delta gene 3 helper were added and the culture incubated at 37° C for 45 minutes without shaking and then at 37° C for 45 minutes with shaking. The culture was centrifuged at 4000 r.p.m. for 10 min. and the pellet resuspended in 2 litres of of 2 x TY containing 100 µg/ml ampicillin and 50 µg/ml kanamycin and grown overnight. Phage was prepared as previously described (PCT/GB91/01134 ex 44).

Panning of the library: Immunotubes (Nunc) were coated overnight in PBS with 4 ml of either 100 µg/ml or 10 µg/ml of recombinant human TNF-α in PBS or 4 ml of 10 µg/ml of Fog-1, a human IgG/k immunoglobulin which recognizes the human red blood cell Rh (D) antigen. Tubes were blocked with 2% Marvel-PBS for 2 hours at 37° C and then washed 3 times in PBS. Approximately 10^{13} TU of phage was applied to the tube and incubated for 30 minutes at room temperature tumbling on an over and under turntable and then left to stand for another 1.5 hours. Tubes were washed 10 times with PBS 0.1% Tween-20 and 10 times with PBS. Phage were eluted by adding 1 ml of 100 mM triethylamine and rotating 15 minutes on an under and over turntable after which the solution was immediately neutralized with 0.5 ml of 1,0 M Tris-HCl, pH 7.4. Phage were then used to infect 10 ml of mid-log *E. coli* TG1 by incubating eluted phage with bacteria for 30 minutes at 37° C. The E. coli were then plated on TYE plates containing 1% glucose and 100 µg/ml ampicillin. The resulting bacterial library was then rescued with delta gene 3 helper phage as described above to prepare phage for a subsequent round of selection. This process was then repeated for a total of 4 rounds of affinity purification with tube-washing increased to 20 times with PBS, 0.1% Tween-20 and 20 times with PBS for rounds 3 and 4.

Characterization of binders: Eluted phage from the 3rd and 4th rounds of selection were used to infect E. coli HB 2151 and soluble scFv was produced (pCANTAB/pIII34 ex 29) from single colonies for assay. In the case of TNF, phage was also rescued from single colonies. ELISAs were performed as previously described with microtitre plates coated with either 10 µg/ml human TNF-α in 50 mM bicarbonate pH 9.6 or 10 µg/ml Fog-1 in PBS. Clones positive in ELISA were further characterized by PCR fingerprinting (pCANTAB/pIII34 ex 20) and then by sequencing.

Results

TNF: Soluble scFv from 1536 colonies and phage from 1152 colonies were screened by ELISA and positive clones were further characterized by PCR fingerprinting and sequencing. In this manner, 15 different binders were identified. Four of these have been partially sequenced (Table 1).

Fog-1: Soluble scFv from 96 clones was screened by ELISA and positive clones were further characterized by PCR fingerprinting and sequencing. In this manner, four different binders were identifed (see Table 1).

Table 1